AUTOPHAGY-RELATED GENE *ATG5* GENETIC VARIANTS AND HEPATITIS B VIRUS INFECTION SUSCEPTIBILITY

SYNOPSIS

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Introduction

Hepatitis B, a global health problem, is a life-threatening liver infection caused by the Hepatitis B virus (HBV). The virus can cause both acute and chronic disease conditions and the latter progresses to cirrhosis, hepatocellular carcinoma and liver failure [1]. Globally, 257 million people are chronically infected with HBV [2] and more than 37 million HBV carriers are in India itself [3]. According to the estimates by World Health Organization (WHO) around 1 lakh people die annually, due to Hepatitis B related complications in India. Although vaccines, antiviral medications, and interferons against HBV are available, however, prediction of the risk, progression or outcome of the infection is required for better management of the disease [4-6]. Moreover, identification of biomarkers that influence HBV treatment response also needs an investigation to develop more effective individualized therapy [7, 8]. For example, recent studies have identified various SNPs in IL28B to be associated with treatment response and viral clearance in Hepatitis C Virus (HCV) infected patients [9-11]

The process of autophagy has emerged as a central component of host defense against diverse viral infections [12]. However, HBV induces autophagy and exploit the process for promoting its own replication [13]. Upregulation of autophagy by HBV has been demonstrated in cell cultures, mouse liver, and in natural infection in a study [14]. Studies suggest, that HBV proteins, X protein (HBx) and small surface protein (SHBs) assist in the induction of the autophagy process and therefore, antiviral drugs that target autophagy could be developed for the treatment of HBV infection [15, 16]. Moreover, any disruption/intervention at initial stages of this pathway could help in tackling the virus replication strategies and resolve the infection.

The process of autophagy initiates in response to stress signals and proceeds with the formation of a double membranous structure, known as the autophagosome. The autophagosome engulfs cytoplasmic constituents and then fuses with the lysosome for degradation of its contents [17, 18]. The process of formation of autophagosome is the most critical phase of the pathway and is regulated by a number of autophagy-related (ATG) genes. Till now, 40 autophagy-related genes have been identified in yeast and most of them have mammalian counterparts [19, 20]. ATG5 is the key autophagy regulatory protein that conjugates to ATG12 and forms a complex with the multimeric protein ATG16L1. The ATG12–ATG5/ATG16L1complex acts as an E3-like enzyme, required for microtubule-associated protein 1 light chain 3 (LC3)–

phosphatidylethanolamine conjugation for autophagosome formation [21, 22]. Liver-specific knockout of the Atg5 gene in transgenic mice has been shown to reduce HBV DNA levels by inhibiting autophagy [14, 23]. In another study, ATG5 levels have been found to be elevated in HBV patients. This elevated level of ATG5 may promote prolonged induction of autophagy process which directly benefits HBV replication [24]. Recently, a number of genetic studies have focused on single nucleotide polymorphisms (SNPs) in autophagy-related genes to study their association with susceptibility to various diseases [25-29]. In this context, SNPs such as rs12212740 and rs510432 of ATG5 have been linked to Asthma susceptibility and lung function [30, 31], rs573775 has been shown to be linked with Systemic lupus erythematosus [32], rs2245214 is found to be associated with Thyroid carcinoma and Paget disease of bone [33, 34]. Moreover, a recent study found a mutation E122D in ATG5 that impairs its interaction with ATG12 and leads to Ataxia development due to autophagy inhibition [35].

Certainly, host genetic factors influence HBV acquisition and play important role in determining progression and outcome of the infection [36, 37]. Evidently, polymorphisms in immunity-related components such as HLA, cytokines, chemokines, interferons and various immunoregulatory factors have been associated with susceptibility and outcome of HBV infection [38]. On the other hand, polymorphisms in autophagy-related genes have not been studied for their association with HBV infection till date.

Identification of disease-associated SNPs in a gene is the first step in population-based association studies. Although the experimental approaches to identify disease-associated SNPs from neutral SNPs provide the best confirmation, these experimental strategies are time-taking and expensive. On the other hand, *in silico* methods provide fast, efficient and cost-effective detection and analysis of deleterious SNPs from a large number of SNPs [39, 40]. Therefore in this study, we have *in silico* explored, polymorphisms in *ATG5* and have genotyped them to find out their association with HBV susceptibility or progression.

Rationale

HBV virus modulates the process of autophagy in the host and exploits it for promoting its own replication. Any disruption/intervention at the initial stages of this pathway could help in virus replication inhibition and infection resolution. Moreover, host genetic factors influence HBV acquisition and may predispose individuals to the infection. ATG5 is an essential autophagy protein that conjugates with ATG12 and ATG16L to form a complex that plays a pivotal role in autophagosome formation *ATG5* deficient mice die within 1 day of birth during early neonatal starvation period highlights its role in regulating the process of autophagy. In this context, polymorphisms in ATG5 could be explored to find out their association with HBV infection susceptibility or progression. The associated SNPs could be used as biomarkers for risk analysis after validation or their impact on treatment response for developing more effective individualized therapies

Considering these lacunae, the following are the objectives of this study.

Objective 1:

To predict the deleterious nsSNPs in the autophagy-related gene *ATG5.*

Objective 2:

To investigate the impact of predicted nsSNPs on the ATG5 protein at sequence as well as structural levels.

Objective 3:

To genotype predicted deleterious nsSNPs as well as selected non-coding SNPs in Hepatitis B Virus-infected patients and healthy individuals.

Objective 1: To predict the deleterious nsSNPs in the autophagy-related gene ATG5.

nsSNPs are located in protein-coding regions and lead to an amino acid substitution in the protein product which could alter the structure, stability, or function of proteins [41]. Subsequently, these polymorphisms could be damaging to the protein structure and function. However, not all nsSNPs are detrimental and lead to disease development. Some nsSNPs, called tolerant nsSNPs, keep up protein function despite the change in protein structure [42]. Therefore, the deleterious nsSNPs could be sorted down from a large number of neutral SNPs using computational tools and further be genotyped to unravel their role in various diseases.

Methodology

The computational methodology followed to achieve this objective is shown in the form of a flow diagram (Fig 1). The amino acid sequence for the ATG5 protein was obtained from NCBI (Protein ID: NP_004840) and UniProt (UniProtKB ID: Q9H1Y0). The SNP information was obtained from NCBI, dbSNP. According to dbSNP, human ATG5 possesses 7,358 Single nucleotide polymorphisms. This SNP dataset was selected as a batch report and submitted to Sorting Intolerant From Tolerant (SIFT) for prediction of nsSNPs [43]. We have used various other *in silico* tools like Polymorphism Phenotype (PolyPhen2) [44], PredictSNP (PolyPhen1, nsSNPAnalyzer, PhD-SNP, and MAPP) [45] to predict the impact of nsSNPs on the ATG5 protein function. The impact of the change in amino acid is predicted as tolerated or damaging/deleterious based on the tolerance index and probability scores. PTM sites of phosphorylation, ubiquitination, palmitoylation, and calpain-cleavage were predicted in the ATG5 protein using various web servers. The NetPhos algorithm was used for the prediction of phosphorylation sites at serine (S), threonine (T) and tyrosine (Y) residues in the ATG5 amino acid sequence. The palmitoylation sites were obtained from CSS-PALM, a tool based on the Clustering and Scoring Strategy (CSS) algorithm for the prediction of palmitoylation sites [46]. An online web server called BDM-PUB (Prediction of Ubiquitination site with Bayesian Discriminant Method; http://bdmpub.biocuckoo.org/) was used to predict ubiquitination sites. Group-based Prediction System-Calpain Cleavage Detector (GPS-CCD) 1.0 tool was utilized for the prediction of calpain-cleavage sites in ATG5 [47].

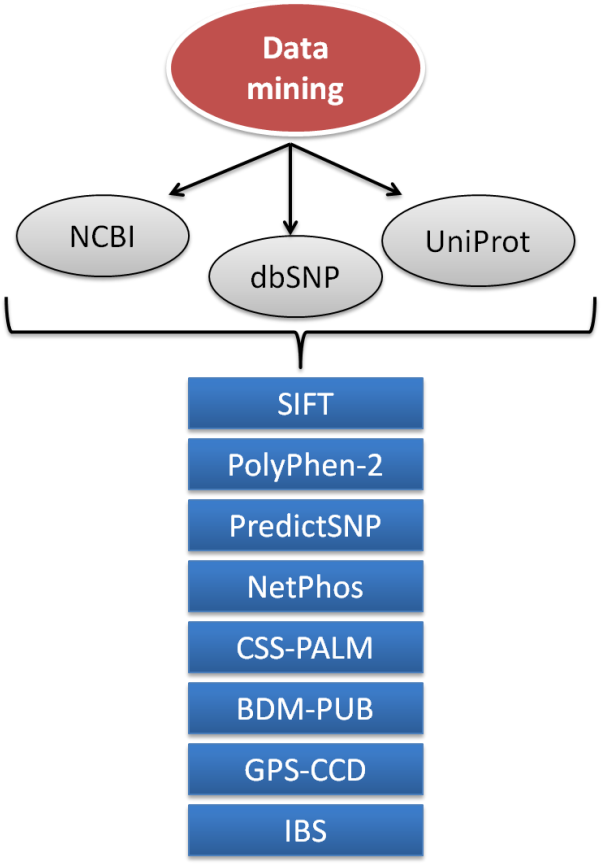

Figure 1: Schematic representation of the computational methodology followed to accomplish this objective.

Results:

- We have identified 3 nsSNPs (rs34793250, M129V; rs77859116, I65V; and rs115576116, A95D) out of which two nsSNPs rs34793250 (M129V) and rs115576116 (A95D) could have damaging/deleterious effects on the ATG5 protein (Table 4). This predicted nsSNPs could be further explored to study their impact on ATG5 protein structure and function.
- Numerous post-translational modification sites were predicted in ATG5. nsSNPs are believed to affect the post-translation modifications like phosphorylation, glycosylation etc. The altered PTM sites by nsSNPs may lead to disruption of protein function by changing its localization, enzymatic activity or even its interactions with other proteins. Therefore, we have predicted various PTM sites in ATG5 and annotated them along with the positions of nsSNPs, over the protein domain structure as shown in Figure 3.

Table 1: The non-synonymous Single Nucleotide Polymorphisms in the coding region of ATG5 with putative damaging effects

SNP INFORMATION		SIFT	PolyPhen-2	PredictSNP			
				PolyPhen-1	nsSNPAnalyzer	PhD-SNP	MAPP
SNP ID	Amino acid change	Predicted impact	Predicted impact	Prediction	Prediction	Prediction	Prediction
rs34793250	M129V	TOLERATED	BENIGN	**Deleterious**	**Disease**	Neutral	**Deleterious**
rs77859116	I65V	TOLERATED	BENIGN	Neutral	Neutral	Neutral	Neutral
rs115576116	A95D	TOLERATED	BENIGN	Neutral	Neutral	**Deleterious**	Neutral

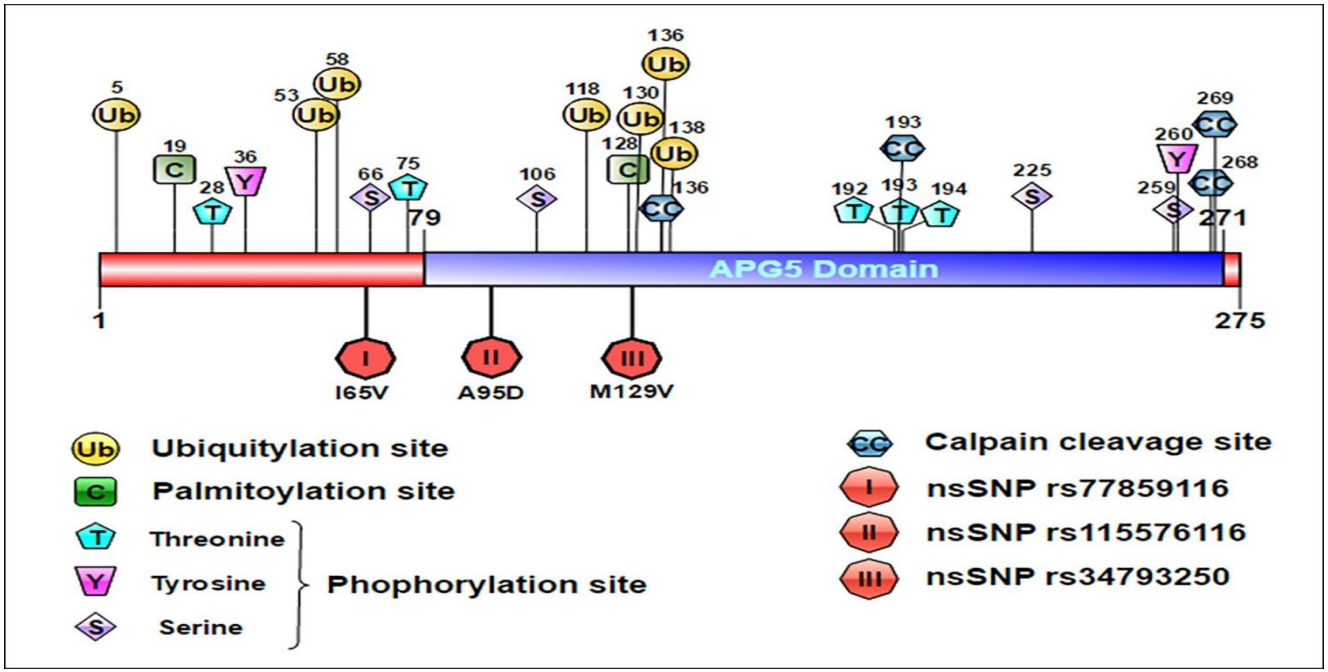

Figure 2: Putative functional sites in the ATG5 protein. Schematic diagram representing the approximate location of the predicted PTMsites (phosphorylation, ubiquitylation, palmitoylation, and Calpain-cleavage sites) and the 3 putative nsSNPs in ATG5.

Conclusion

We have predicted 3 nsSNPs in ATG5 gene (rs34793250, rs77859116, and rs115576116) and based on *in silico* analysis, among these, two are deleterious (rs34793250, rs115576116) that might have a damaging impact on protein structure and ultimately contribute to an individual's susceptibility to various diseases. We selected these nsSNPs for studying their impact on ATG5 protein (both at sequence and structural level)

Objective 2: To investigate the impact of predicted nsSNPs on the ATG5 protein at sequence as well as structural level.

Because nsSNPs occur in the coding region and may lead to amino acid substitution in the corresponding protein, they are considered to be more significant [48]. Mutations that result in gain/loss of structural and functional properties of the protein may prove to be pathogenic or disease associated. Moreover, they may affect the stability of the protein structure which may further affect its functions [49]. In order to analyze the impact of predicted nsSNPs on ATG5 protein structure and function, we followed a computational methodology as described below.

Methodology

The computational methodology followed to achieve this objective is shown in Figure 3. The protein sequence for human ATG5 was retrieved from the National Center for Biotechnology Information (NCBI) (Accession: NP_004840) and the corresponding 3D protein structure from Protein Data Bank DB ID: 4GDK). The methodology was dived into two main segments; Sequence-based analysis and Structure-based analysis. Sequence-based analysis was carried out for pathogenicity and stability analysis whereas the structure based analysis was performed for conservation, stability of mutated protein structure, for predicting structural variations of the protein complex upon mutation, computation of total energy, and prediction of binding affinity change upon mutations

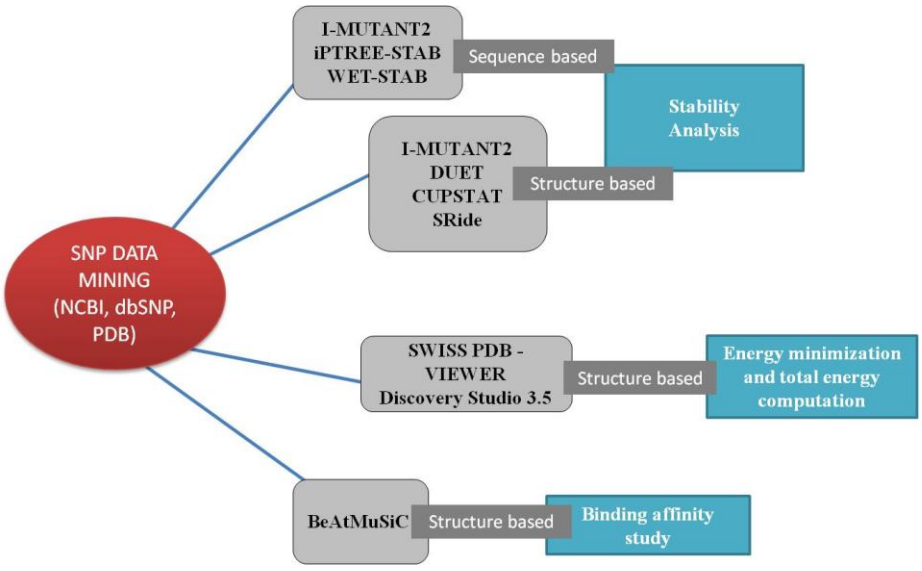

Figure 3: Diagrammatic representation of the methodology followed and the computational tools used to accomplish this objective.

Results:

- The nsSNPs showed a decrease in stability of ATG5 both at sequence (Table 2) and at the structural level (Table 3) as predicted by various *in silico* tools.

Table 2. Prediction of change Gibbs free energy upon mutations from protein sequence using three different tools, I-MUTANT2, iPTREE-STAB, and WET-STAB.

ATG5 nsSNP	**I-MUTANT2** ΔΔG Value (kcal/mol)	**iPTREE-STAB** ΔΔG Value (kcal/mol)	**WET-STAB** ΔΔG Value (kcal/mol)	**PREDICTION**
I65V	-1.53	-1.3200	-1.005	**Destabilizing**
A95D	-0.01	-1.1814	-0.312	**Destabilizing**
M129V	-1.05	-1.6632	-0.312	**Destabilizing**

ΔΔG <0: Decrease Stability; ΔΔG >0: Increase Stability

Table 3. Prediction of change Gibbs free energy upon mutations from protein structure by using three different tools, I-MUTANT2, DUET and CUPSTAT.

ATG5 Mutations (4GDK structure)	**I-MUTANT2**	**DUET**			**CUPSTAT**	**PREDICTION**
	ΔΔG Value (kcal/mol)	mCSM ΔΔG Value (kcal/mol)	SDM ΔΔG Value (kcal/mol)	DUET COMBINED ΔΔG Value (kcal/mol)	ΔΔG Value (kcal/mol)	
I65V	-0.50	-1.024	-0.02	-1.005	-1.31	**Destabilizing**
A95D	-1.10	-1.064	-1.44	-1.046	-2.37	**Destabilizing**
M129V	-1.16	-1.826	-1.6	-1.865	-0.69	**Destabilizing**

ΔΔG <0: Decrease Stability; ΔΔG >0: Increase Stability

- The nsSNPs destabilized the ATG5 complex structure as they were found to increase the total energy of the complex as shown in Figure 4

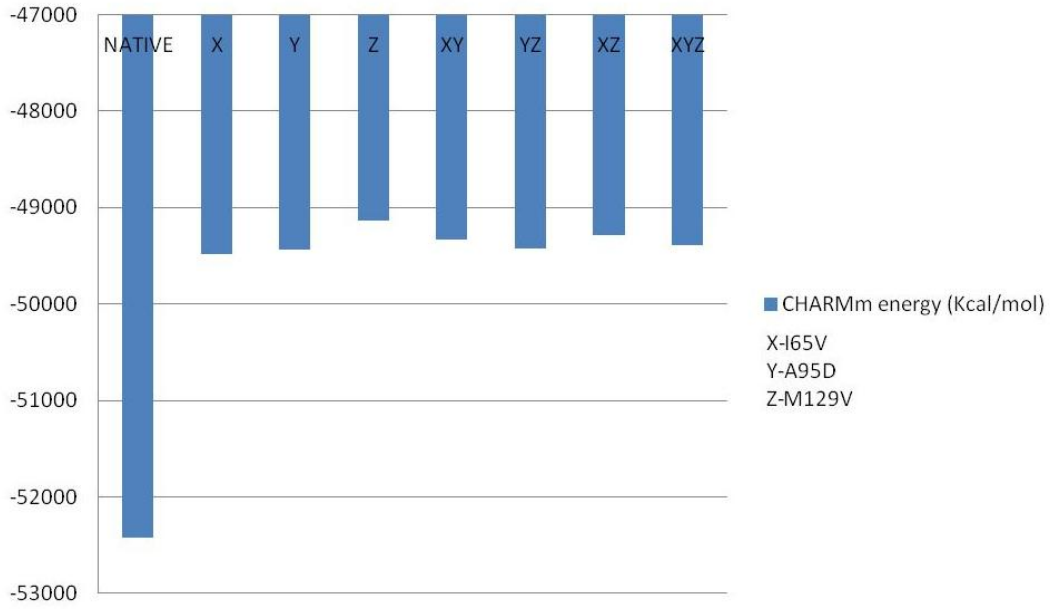

Figure 4: Graphical representation of total energy computation of native as well as mutant structures, done by applying CHARMm force field. X, Y, and Z represent I65V, A95D and M129V, respectively. Energy calculations were also done for their different combinations (XY, YZ, XZ, and XYZ).

- The nsSNPs were found to decrease the binding affinity of ATG5 with ATG12 and ATG16 as shown in Table 4

Table 4: The binding energy change observed upon mutations predicted by BeAtMusiC tool.

	BeAtMuSiC		
4GDK	ΔΔGBind (kcal/mol)	Interface	Prediction
Chain AD (ATG12) + Chain BE(ATG5)			
I65V	0.31	No	Decreases binding affinity
A95D	0.37	No	Decreases binding affinity
M129V	0.45	No	Decreases binding affinity
Chain BE (ATG5) + Chain CF(ATG16L1)			
I65V	0.31	No	Decreases binding affinity
A95D	0.84	Yes	Decreases binding affinity
M129V	0.43	No	Decreases binding affinity

Conclusion

Sequence and structure-based analysis of the nsSNPs (rs34793250, rs77859116, and rs115576116), predicted in the first objective, indicated the degree of destabilization caused, based on decreased Gibbs free energy change values obtained from different tools. Further, these nsSNPs resulted into loss or replacement of stabilizing residues in the protein complex. Though all the predicted nsSNPs increased the total energy of the complex, the mutation M129V was observed to increase the energy to the maximum. Furthermore, we predicted the change in binding affinity of ATG5 towards ATG12 and ATG16L1 proteins. It was observed that all the mutations lead to an overall decrease in binding affinity of ATG5 with ATG12 and ATG16L1. On the whole, all the results from different parameters indicate that these mutations in ATG5 are likely to have a potentially damaging impact over the protein as well as its complex structure and could play an important role in various diseases. Therefore, we selected these nsSNPs for genotyping in HBV infected patients and healthy controls to look for their association with the disease susceptibility, progression or outcome.

Objective 3: To genotype predicted deleterious nsSNPs as well as selected non-coding SNPs in hepatitis B virus-infected patients and healthy individuals.

Apart from nsSNPs, we also selected three other important non-coding SNPs of ATG5 i.e. rs2245214, rs12212740 and rs510432 to look for their association with HBV infection susceptibility. These SNPs of ATG5 have already been shown to be associated with Asthma [30, 31], Thyroid carcinoma [33] and Paget disease of bone [34]. Primers were designed for each SNP and RFLP was carried out to genotype these SNPs in the HBV infected as well as healthy control samples.

Methodology

Study population

The study group comprised of 800 samples (550 HBV infected patients and 250 healthy individuals) from North India.

Table 5: Clinical and demographic features of HBV patients and control group**.**

Characteristerics	**HBV patients (550)**	**Control group (250)**
Gender		
Male (count)	330	139
Female (count)	220	111
Age (Average)	18-75 (37.02)	22-69 (32.6)
Serological and biochemical parameters		
HBsAg	+	-
HCV	-	-
HIV	-	-
AST(IU/L)	100±79	28±10
ALT(IU/L)	105±89	30±12
Clinical diagnosis (Infection stage)		
Asymptomatic carrier	140	-
Acute hepatitis	114	-
Chronic hepatitis	256	-
Liver cirrhosis	40	-

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.(Mean±SD)

Sampling and DNA isolation

Peripheral blood (2ml) was collected from the HBV patients and healthy individuals in vacutainer tubes with anticoagulant (EDTA). Genomic DNA was extracted from the blood sample using a standard salting out procedure [50].

PCR primers and restriction enzymes used for genotyping

Specific amplification primers were designed for detection of the selected SNPs. Such primers were able to amplify the target sequence containing restriction site at the SNP position. Gradient PCR was performed for each primer set to select the most optimum annealing temperature for the amplification of target sequence containing the selected SNP. The primer sequences, annealing temperatures and restriction enzymes used for genotyping all six SNPs of ATG5 are shown in Table 6.

Briefly, total 25µl PCR reaction was prepared that included, 12.5 µl PCR master mix (Promega, Madison, US), 0.25µM of each primer and 20ng of DNA template. Amplified PCR products were analyzed on 1.5% agarose gel with ethidium bromide. For RFLP analysis, PCR products were digested with 1-3 Units of the respective restriction enzymes and were incubated at 37 °C, overnight. Digested products were analyzed on 2.5-3% agarose gel containing ethidium bromide for determining different genotypes for respective SNPs.

Statistical analysis

Genotype data was compiled and the allelic and genotypic frequencies for all the SNPs were calculated. Odds ratios (OR) and 95% confidence intervals (95%CI) were calculated to assess the risk associated with alleles and genotypes. MEDCALC software (MedCalc, Ostend, Belgium) was used to calculate an odds ratio, class interval and *p-value* for significance level. Virological (viral load) and biochemical data (AST and ALT levels), represented as Mean±Standard deviation, was assessed by One-way Analysis of Variance (ANOVA) and t-tests using GraphPad Prism 7 software. p-value < 0.05 was considered statistically significant.

Table 6: Primer used for PCR-RFLP analysis to genotype ATG5 polymorphisms.

ATG5 SNP	Type	Primer sequences 5'– 3'	Annealing temperature (°C)	Amplified product size (bp)	Restriction enzyme	Genotypes (bp)
rs34793250 T/C [M129V]	Missense variant	Forward primer $A_{(15)}C_{(15)}$ TGTTTTAAAGCATCAGCTTCTTTCg Reverse primer TGGGTTATTTCAGTGCTAAGAGA	57	254	*RsaI*	TT-198, 56 TC-254, 198, 56 CC-254
rs77859116 T/C [I65V]	Missense variant	Forward primer $C_{(15)}A_{(15)}$ CCTTCATATTCAAACCATATCTCAtTA Reverse primer TATTCAAAATAATGCAAAGAACACGG	56	259	*AseI*	TT-204, 55 TC-255, 204, 55 CC-259
rs115576116 G/T [A95D]	Missense variant	Forward primer AATTGGGGGAGGGGAACAGA Reverse primer AAACCACCTGATGCCTGGAAA	64	966	*BglII*	GG-966 GT-966, 729, 237 TT-729, 237
rs2245214 C/G	Intronic variant	Forward primer $C_{(12)}A_{(7)}$CACCCCCA GCTTTTAAATTTTTATATGAACTTATCAGTcGA Reverse primer GCTCCTAGCCCAGGTTTAGA	60	518	*SalI*	CC-518 CG-518, 462, 56 GG-462, 56
rs12212740 G/A	Intronic variant	Forward primer $C_{(15)}$ $AAGA_{(6)}$ CCGCCG ATTTTTAAACAAAATGTTAGCAGcT Reverse primer AACCTTGGGCAAGTTATTTGA	52	557	*PvuII*	GG-504, 53 GA-557, 504, 53 AA-557
rs510432 G/A	Intronic variant	Forward primer $A_{(15)}C_{(15)}$TCCAACAAAGTAGAGAAGAAGATCAtAT Reverse primer TCTACCCTCTTCTGAGAATCTTG	56	260	*NdeI*	GG-205, 55 GA-260, 205, 55 AA- 260

*The mismatch base is shown in a lower case in the forward primer.

Results and Discussion

The genotypes of different SNPs were analyzed by agarose gel electrophoresis. The representative gel picture for each SNP is shown in Figure 5 and Figure 6. All the SNPs were successfully genotyped, however, we did not observe all the three genotypes for two nsSNPs that are rs77859116 T/C and rs115576116 G/T. Therefore, these two SNPs were analyzed in a total of 300 samples only.

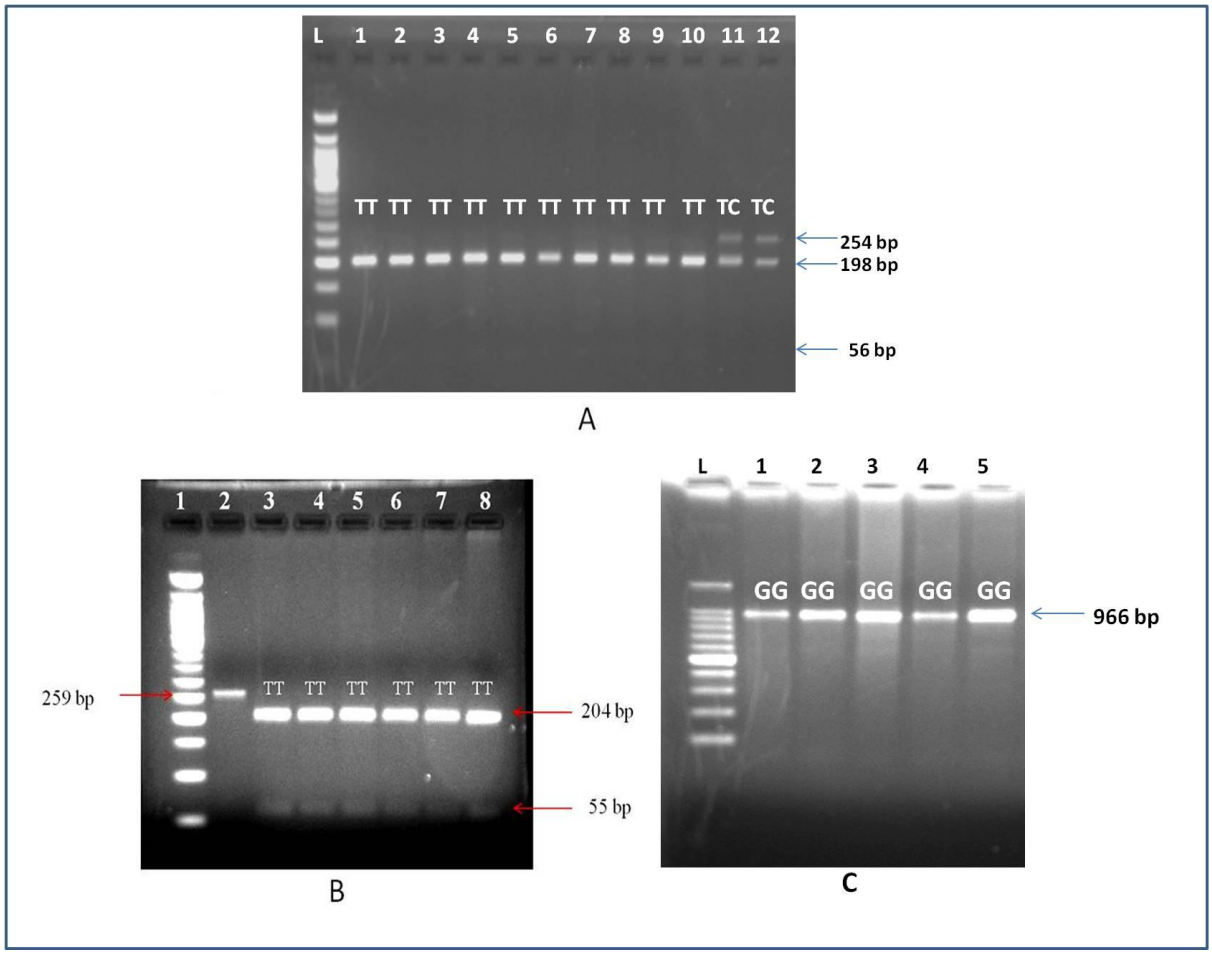
L 1 2 3 4 5 6 7 8 9 10 11 12
TT TT TT TT TT TT TT TT TT TT TC TC
254 bp
198 bp
56 bp
A
1 2 3 4 5 6 7 8
259 bp
TT TT TT TT TT TT
204 bp
55 bp
B
L 1 2 3 4 5
GG GG GG GG GG
966 bp
C

Figure 5: Representative image for different genotypes obtained after PCR-RFLP analysis of nsSNPs of ATG5. A. rs34793250 T/C [M129V] PCR amplification (L: 50bp ladder; Lane 1-Lane 12: HBV-infected samples; B. rs77859116 T/C [I65V] RFLP analysis (Lane 1: 50bp ladder, Lane 2-Lane 8: HBV infected samples); C. rs115576116 G/T [A95D] RFLP analysis (Lane 1: 100bp ladder, Lane 1-Lane 5: HBV infected samples)

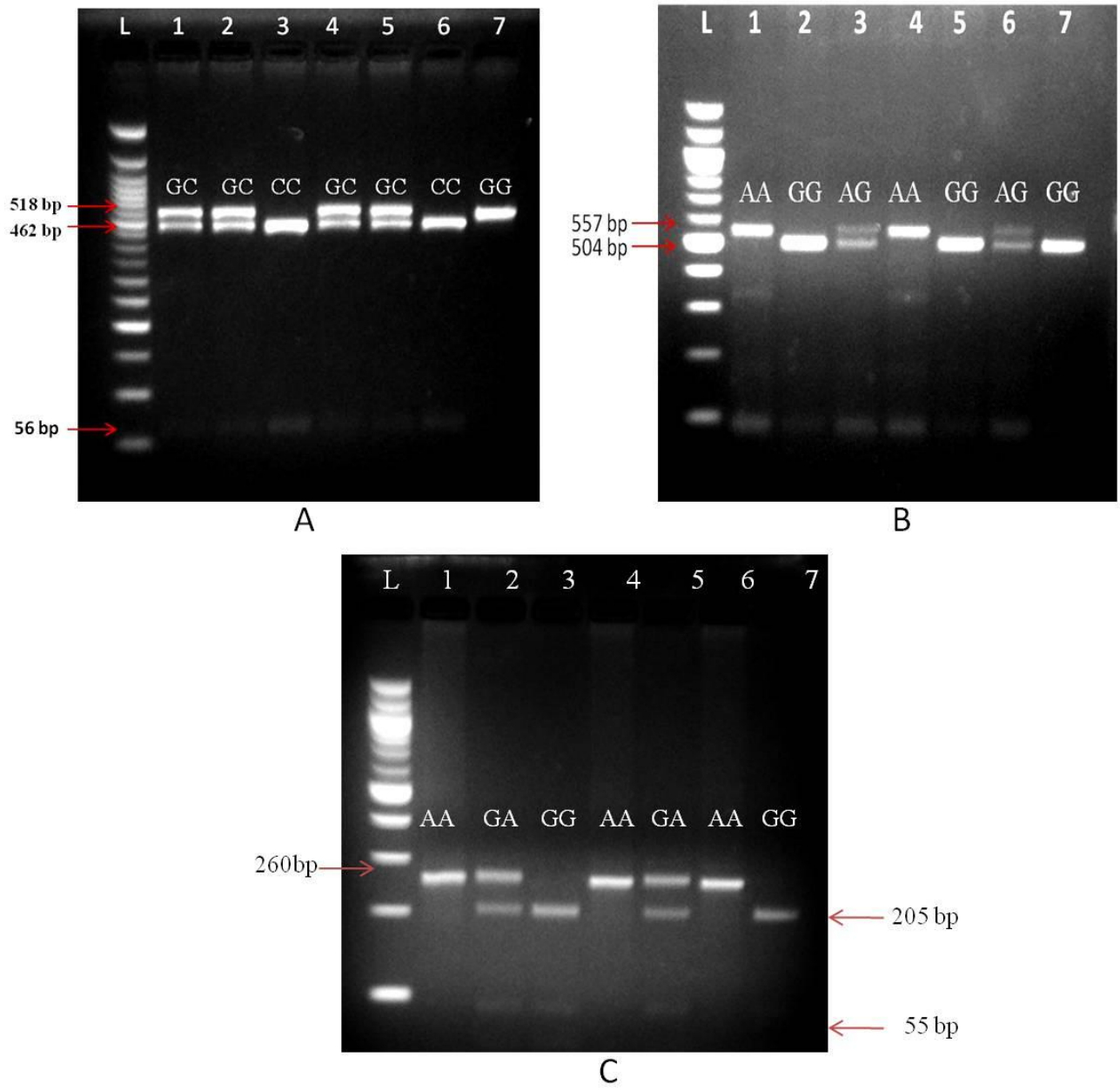

Figure 6: Representative image for different genotypes obtained after PCR-RFLP analysis of non-coding SNPs of ATG5. A. rs2245214 C/G RFLP analysis (L: 50bp ladder; Lane 1-Lane 7: HBV-infected samples); B. rs12212740 G/A RFLP analysis (L: 100bp ladder, Lane 1-Lane 7: HBV infected samples); C. rs510432 A/G RFLP analysis (L: 100bp ladder, Lane 1-Lane 7: HBV infected samples).

- The genotypic and allelic frequencies for all the SNPs were calculated and tabulated (Table 7-8). The genotypic and allelic frequencies were also calculated in different stages of HBV infection and compared with healthy control samples. Furthermore, the viral load, AST, and ALT levels were compared with the different genotypes of the SNPs in HBV infected patients. However, tabulated data for this analysis is shown only for the nsSNP rs34793250 (T>C) in Table 9-10.

- The nsSNP rs34793250 (T>C) of ATG5 was found to be associated with HBV susceptibility. The C allele was found to be significantly associated with HBV risk (T vs. C, Odds ratio (OR) =3.35, 95% CI=1.31-8.59, p=0.01; TT vs. TC, OR= 3.4, 95% CI=1.33-8.85, p=0.01) (Table 7 and 8). Moreover, we found a significant association of C allele in the asymptomatic, acute and chronic stages of HBV infection whereas no association of this SNP was found in cirrhotic patients (Table 9). Furthermore, viral load in different HBV infection categories was co-related with the genotypes of HBV patients. We did not find any significant difference in mean viral load between the host genotypes for this SNP. Although not significant, the low viral load was observed in patients with heterozygous genotype TC as compared to homozygous wild-type genotype TT, in all categories of HBV infection (Table 10). Additionally, statistical analysis was carried out to study the relation between the patient genotypes and biochemical parameters namely, AST and ALT (Table 10). The mean AST level was found to be significantly high in patients (chronic stage) with heterozygous genotype (TC) than with homozygous wild-type genotype (TT vs. TC, p= 0.02). The same pattern was observed for ALT levels where the patients (asymptomatic and chronic stage) with heterozygous genotype (TC) were found to have high mean ALT level as compared to patients with homozygous wild-type genotype (TT vs. TC, p= 0.02) (Table 10).

Table 7: Genotypic frequency of ATG5 polymorphisms in HBV cases and healthy control samples. (N = total number of samples)

ATG5 SNP rs ID Genotype and allele	HBV (N=550)	Control (N =250)	Odds ratio [95% CI]	p-value
rs34793250 (T>C)				
TT	514[93]	245[98]	Ref.	\
TC	36[7]	5[2]	**3.43** [1.3303 to 8.8538]	**0.01**
CC	0[0]	0[0]		
rs77859116 (T>C)				
TT	150/150[100]	150/150[100]		
TC	0/150[0]	0/150[0]	Not estimable	Not estimable
CC	0/150[0]	0/150[0]		
rs115576116 (G>T)				
GG	150/150[100]	150/150[100]		
GT	0/150[0]	0/150[0]	Not estimable	Not estimable
TT	0/150[0]	0/150[0]		
rs2245214 (C>G)				
CC	197[36]	107[43]	Ref.	\
CG	275[50]	121[48]	1.23 [0.8982 to 1.6966]	0.19
GG	78[14]	22[9]	**1.93** [1.1352 to 3.2666]	**0.02**
CG+GG	353[64]	143[57]	1.34 [0.9881 to 1.8193]	0.06
CG+CC	472[86]	228[91]	Ref.	\
GG	78[4]	22[9]	**1.71** [1.0400 to 2.8204]	**0.03**
rs12212740 (G>A)				
GG	500[90.91]	232[92.8]	Ref.	\
GA	48[8.73]	17[6.8]	1.13 [0.6314 to 2.0322]	0.68
AA	2[0.36]	1[0.4]	0.92 [0.0827 to 10.1642]	0.94
GA+AA	50[9.09]	18[7.2]	1.12 [0.6338 to 1.9818]	0.67
GA+GG	548[99.64]	249[99.6]	Ref.	
AA	2[0.36]	1[0.4]	0.91 [0.0820 to 10.0693]	0.94
rs510432 (G>A)				
GG	75[13.64]	51[20.4]	Ref.	\
GA	388[70.54]	159[63.6]	**1.66** [1.1115 to 2.4774]	**0.013**
AA	87[15.82]	40[16]	1.48[0.8824 to 2.4790]	0.136
GA+AA	475[86.36]	199[79.6]	**1.62** [1.0964 to 2.4030]	**0.015**
GA+GG	463[84.18]	210[84]	Ref.	\
AA	87[15.82]	40[16]	0.99 [0.6557 to 1.4841]	0.948

p-value <0.05 was considered significant. Significant values are shown in bold.

Table 8: Allele frequencies of ATG5 polymorphisms in HBV cases and healthy control samples. (N = total number of samples)

ATG5 SNP rs ID Genotype and allele	**HBV (N=550)**	**Control (N =250)**	**Odds ratio [95% CI]**	**p-value**
rs34793250 (T>C)				
T	1064 [97]	495 [99]	Ref.	\
C	36[3]	5 [1]	**3.35** [1.3065 to 8.5878]	**0.01**
rs77859116 (T>C)				
T	300[100]	300[100]		
C	0 [0]	0 [0]	Not estimable	Not estimable
rs115576116 (G>T)				
G	300[100]	300[100]		
T	0 [0]	0 [0]	Not estimable	Not estimable
rs2245214 (C>G)				
C	669[61]	335[67]	Ref.	\
G	431[39]	165[33]	**1.30** [1.0473 to 1.6336]	**0.02**
rs12212740 (G>A)				
G	1054[96]	481 [96]	Ref.	\
A	46 [4]	19 [4]	1.10 [0.6405 to 1.9060]	0.72
rs510432 (G>A)				
G	538[49]	261[52]	Ref.	\
A	562[51]	239 [48]	1.14[0.9232 to 1.4096]	0.22

p-value <0.05 was considered significant. Significant values are shown in bold.

Table 9: Genotypic and allelic frequencies of ATG5 nsSNP rs34793250T/C in different HBV infection stages and healthy control.

HBV infection Categories (no of samples)	Genotype and Allele	Cases (N=550)	Control (N=250)	Odds ratio [95% CI]	p value
ASYMPTOMATIC (140)		n/N	n/N		
	TT	129/140	245/250	Ref.	\
	TC	11/140	5/250	**4.18** [1.42 to 12.28]	**0.01**
	CC	0/140	0/250		
	T	269 (96.07%)	495 (99%)	Ref.	\
	C	11 (3.93%)	5(1%)	**4.05** [1.39 to 11.77]	**0.01**
ACUTE (114)	TT	106/114	245/250	Ref.	\
	TC	8/114	5/250	**3.70** [1.18 to 11.57]	**0.02**
	CC	0/114	0/250		
	T	220 (96.49%)	495 (99%)	Ref.	\
	C	8 (3.51%)	5 (1%)	**3.6** [1.16 to 11.13]	**0.03**
CHRONIC (256)	TT	241/256	245/250	Ref.	\
	TC	15/256	5/250	**3.05** [1.09 to 8.52]	**0.03**
	CC	0/256	0/250		
	T	497 (97.07%)	495 (99%)	Ref.	\
	C	15 (2.93%)	5 (1%)	**2.99** [1.08 to 8.28]	**0.04**
CIRRHOSIS (40)	TT	38/40	245/250	Ref.	\
	TC	2/40	5/250	2.58 [0.48 to 13.77]	0.27
	CC	0/40	0/250		
	T	78 (97.5%)	495 (99%)	Ref.	\
	C	2 (2.5%)	5 (1%)	2.53 [0.48 to 13.31]	0.27

p-value <0.05 was considered significant. Significant values are shown in bold.

Table 10: Association of virological (viral load) and biochemical parameters (AST, ALT) with rs34793250 (T>C) the genotypes of HBV infected patients.

HBV infection category (no of samples)	Genotypes (no of samples)	Viral load (copies/ml) [Mean±SD]	p value	AST (IU/L) [Mean±SD]	p value	ALT (IU/L) [Mean±SD]	p value
Asymptomatic (140)	TT (129) TC (11) CC(0)	130±111 77± 97 0	0.13*	60±45 48±20 0	0.38*	46±51 99±190 0	**0.02***
Acute (114)	TT (106) TC (8) CC(0)	1624±1459 834±429 0	0.13*	69±51 63±32 0	0.74*	87±59 71±36 0	0.45*
Chronic (256)	TT (n=241) TC (n=15) CC(n=0)	2151191±3710419 983546±1364270 0	0.23*	102±61 156±281 0	**0.02***	104±72 166±319 0	**0.02***
Cirrhosis (40)	TT (38) TC (2) CC(0)	1890032059 ±10152697922 45676579±0.71 0	0.80*	168±75.3 118±54 0	0.36*	154±85 138±26 0	0.79*

*Student t-test (TT vs. TC), p-value <0.05 was considered significant (shown in bold),
ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

- Similarly, the non-coding SNP rs2245214 (C>G) was also found to be associated with HBV susceptibility. The G allele was found to be significantly associated with HBV risk as compared to control samples (C vs. G, OR=1.30, 95%CI= 1.0473 to 1.6336], p=0.02) (Table 8). Also, we observed significant association in the homozygous (CC vs. GG, OR=1.93, 95%CI=1.14 to 3.27, p=0.02) and recessive genotype models (CC+CG vs. GG, OR= 1.71, 95%CI=1.04-2.82, p=0.03). Moreover, the SNP was found to be significantly associated in the chronic (C vs. G, OR=1.36, 95%CI=1.05 to 1.75, p=0.02; CG+CC vs. GG, OR=1.94, 95%CI=1.12 to 3.37, p=0.01; CC vs. GG, OR=2.20, 95%CI=1.22 to 3.95, p=0.01) and cirrhosis stage (C vs. G, OR=1.66, 95%CI=1.03 to 2.68, p=0.03; CC vs. GG, OR=3.10, 95%CI=1.08 to 8.87, p=0.04) of HBV infection while there was no association found in the asymptomatic and acute stages of infection. Also, we observed a wide variation in average viral load in patients with different genotype in all categories of HBV infection. In asymptomatic stage, mean viral load was found to be low, while in acute, chronic and cirrhosis stages, we found higher mean viral load in patients with heterozygous (CG) and homozygous mutant genotype (GG). However, we found significant difference in mean viral load in patients only at the cirrhosis stage of HBV infection between the different genotypes (CC vs. CG, 169603285±188534786 [n=11] vs. 3357082456±3796674214 [n=22], p= 0.01; CC vs. GG, 169603285±188534786 (n=11) vs. 2522638024±1881569975 (n=7), p<0.001; CC vs. CG+GG, p<0.001).

- No significant association was observed for the promoter SNP rs510432 (G>A), in the allelic model of polymorphism (Table 8). However, the genotypic frequencies reveal its association with HBV susceptibility in the heterozygous (GG vs. GA, OR=1.66, 95%CI=1.1115- 2.487, p=0.01) and dominant model (GG vs. GA+AA, OR=1.62, 95%CI=1.10- 2.40), p=0.02) of polymorphism (Table 7). This promoter SNP was also found to be significantly associated in asymptomatic (heterozygous model, OR=2, 95%CI=1.10 to 3.65,p=0.02;dominant model, OR=1.85, 95%CI=1.025 to 3.36, p=0.04) and in chronic stage (GG vs. GA, OR=1.76, 95%CI=1.08 to 2.86,p=0.02; GG vs. GA+AA, OR=1.74, 95%CI=.1.08 to 2.82, p= 0.02) of HBV infection while no association was found in the acute and cirrhosis patients. Also, we found a significant difference in the mean viral load of patients with homozygous wild-type genotype (GG) and homozygous mutant genotype (AA) in the asymptomatic category (GG vs. AA, 80.5±71 [n=17] vs. 161±77 [n=17], p=0.004). However, we did not observe a similar trend in other categories (chronic and cirrhosis) of HBV infection, where the higher viral load was found in the wild-type genotype (GG) of patients as compared to the mutant genotype (AA). Also, we did not observe any significant difference in AST and ALT levels with respect to different genotypes of patients.

- We did not observe any significant association for rs77859116 (T>C), rs115576116 (G>T), rs12212740 (G>A) SNPs of ATG5 with HBV infection susceptibility (Table 7 and 8). However, for rs12212740 we observed significant difference in the mean viral

load of patients with the wild type (GG) and mutant genotype (AA) of the patient (acute stage). The average viral load was observed to be higher in the patient with homozygous wild type genotype (GG vs. AA, 1802± 1614 [n=105] vs. 604±126 [n=8], p=0.03; GG vs. GA+AA, 1802±1614 [n=105] vs. 600±118 [n=9], p=0.04). Moreover, the patients with GA genotype in the acute stage of infection had higher mean ALT (GG vs. GA, 66±34 [n=105] vs. 107±142 [n=8], p= 0.02) and mean AST levels (GG vs. GA, 84±44 [n=105] vs. 128±147 [n=8], p= 0.04).

- Therefore, these ATG5 SNPs (nsSNPs rs34793250 [M129V] (T/C), and non-coding SNPs rs2245214 (C/G) and rs510432 (G/A)) could be further explored and investigated in another population set to validate their contribution in HBV infection progression, outcome or in treatment response.

Conclusion

The predicted deleterious nsSNPs by *in silico* tools, as well as the other non-coding SNPs of ATG5, were successfully genotyped in 550 HBV patients as well as in 250 healthy controls. We found a significant association of the most destabilizing nsSNP rs34793250 (T/C) [M129V] with HBV infection susceptibility. According to the observed allelic frequency, the C allele predisposes individuals to HBV infection. Moreover, the SNP was found to be significantly associated with different HBV infection stages (asymptomatic, acute and chronic). The TC genotype of this SNP was also found to be significantly associated with higher mean AST and ALT levels in patients. SNP rs2245214 (C/G), located in the non-coding region, was also found to be significantly associated with HBV infection susceptibility. Thus, the presence of G allele in the genotype of an individual may increase the risk for HBV acquisition. The third SNP, rs510432 (G/A) located in the promoter region of ATG5, did not show any association with HBV susceptibility in the allelic model. However, a significant association has been observed in the genotypic models (heterozygous and dominant), demonstrating the risk imposed by the mutant allele. Moreover, the patients with mutant AA genotype had significantly higher mean viral load. The SNP rs12212740 (G/A) does not play any role in HBV infection as no significant association was found in any of the genetic models and stages of infection. However, the patients with genotype GG were found to have higher mean viral load and the genotype GA has higher ALT and AST levels which again point towards the significance of this SNP in HBV infection.

In summary, the study highlights the important SNPs rs34793250 (T/C), rs2245214 (C/G) and rs510432 (G/A) in ATG5 which increase individuals' risk of HBV infection in our population. We have already shown the deleterious impact of the nsSNP rs34793250 over ATG5 protein structure and function through an *in silico* approach. The *in silico* methodology, therefore, helped in the selection of an important nsSNP in ATG5 which may increase HBV infection susceptibility in our study population. The other SNP rs2245214 which is significantly associated with HBV infection has been already linked to Paget disease of bone and thyroid carcinoma. Moreover, this polymorphism has been shown to result in loss of recognition site for SRp40 protein which is involved in mRNA splicing. Last but not the least, SNP rs510432, located in the promoter region of ATG5 gene, also plays a substantial role in predisposing individuals towards HBV infection. Moreover, this SNP constitutes the binding site for two transcription factors STAT1and C-Fos and therefore it may lead to transcriptional aberrations that could lead to disease development. Consequently, these three SNPs of ATG5 have considerable implications and significance, in addition, to being involved in HBV infection susceptibility. Hence they must be further genotyped and analyzed in more number of samples and in other population sets before utilizing them as markers for HBV infection susceptibility.

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Publications

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Conference posters

Avni Vij and Harish Changotra, *"In silico prediction and analysis of non synonymous single nucleotide polymorphisms (nsSNPs) in Autophagy-related gene 5 (ATG5)"*at National Conference on Recent Trends in Biomedical Engineering, Cancer Biology, Bioinformatics and Applied Biotechnology (BECBAB-2015) in November, 2015 at Jawaharlal Nehru University, New Delhi.

Avni Vij and Harish Changotra, "*Role of Autophagy-related gene 5 (ATG5) single nucleotide polymorphism rs2245214 (C/G) with HBV susceptibility in North Indian population",* at International Conference on Recent Research in Biomedical Engineering, Cancer Biology, Stem Cells, Bioinformatics and Applied Biotechnology (BECBAB-2017) in February, 2017 at Jawaharlal Nehru University, New Delhi.